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14. ABSTRACT
The metabolic consequences of obesity may be critical in the development of ovarian cancer (OC), resulting in biologically different cancers than those that arise in leaner women. This may occur through aberrant modulation of mTOR signaling, given that alterations in this pathway are common in both obesity and OC. We found that OCs arising in obese *versus* lean mice and women have distinct gene expression profiles, involving many metabolically relevant genes and pathways. In addition, diet induced-obesity promoted tumor growth in a genetically engineered mouse model of OC, coincident with mitochondrial dysfunction and energy supplied by fatty acid oxidation rather than glycolysis in tumors from obese *versus* lean mice. Metformin (AMPK activator) but not everolimus (mTOR inhibitor) was more efficacious in the inhibition of tumor growth in obese *versus* lean mice. Metformin's increased efficacy in the obese setting corresponded with inhibition of mitochondrial complex 1, halting of fatty acid oxidation and stimulation of glycolysis in only tumors from obese mice. For our *in vitro* studies, metformin and everolimus had similar effects on proliferation, inhibition of mTOR signaling and glycolysis but opposite effects on glucose uptake, which may also contribute to metformin's enhanced anti-tumorigenic effects in the setting of obesity.

15. SUBJECT TERMS
Ovarian cancer, mTOR pathway, mTOR inhibitors, metformin, obesity, genomics, metabolomics

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(1) INTRODUCTION

Obesity leads to elevated incidence and worse outcomes for ovarian cancer (OC) (1-18). We postulate that the metabolic consequences of obesity may be crucial in the development of OC, resulting in biologically different cancers than those that arise in normal weight women. This may occur through aberrant modulation of mTOR signaling, given that alterations in this pathway are common in both disease processes. Thus, obese OC patients may derive increased benefit from chemotherapeutic agents related to inhibition of this pathway, such as mTOR inhibitors or metformin. This proposal will address this question by investigating the impact of obesity on the proliferative and metabolic effects of mTOR inhibitor and metformin treatment in three model systems: *in vitro* using OC cell lines; *in vivo* using a novel serous ovarian tumor murine model; and in a pilot clinical trial. The role of obesity in OC initiation and promotion will be evaluated through comprehensive cross-species genomic and metabolomic analysis with the goal of identifying common genetic or metabolic biomarkers associated with obesity-driven cancers and differential response to treatment in the obese and non-obese state. If our hypothesis is true, optimization of OC treatment may need to encompass tumor characteristics as well as obesity status.

(2) KEYWORDS: Ovarian cancer, mTOR pathway, mTOR inhibitors, metformin, obesity, genomics, metabolomics

(3) OVERALL PROJECT SUMMARY

Task 1 (Aim 1): To assess the effect of the mammalian target of rapamycin (mTOR) inhibitor everolimus and metformin on key metabolic pathways in human ovarian cancer cell lines under high and low glucose conditions.

Overweight and obese states may be linked to OC through nutrient-sensitive signaling cascades, such as the insulin/insulin growth factor (IGF) and PI3K/Akt/mTOR pathways (19-23). Hyperinsulinemia, insulin growth

Figure 1. Metformin and everolimus increased phosphorylation of GSK-3β and GSK-3α and reduced phosphorylation of S6 expression in a dose dependent manner in the SKOV3 cell line after 24 hours of treatment, as assessed by Western immunoblotting analysis. In parallel, metformin and everolimus increased GLUT1 and GLUT4 expression. Similar effects were seen in the IGROV1 and OV5 cell lines.

factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) levels are important in OC development and progression in experimental and epidemiological studies (24-27). Signaling through IGF-1R leads to activation of the PI3K/Akt/mTOR pathway, and components of this pathway are often mutated, amplified or aberrantly expressed in OCs (28-33). Thus, mTOR inhibitors, such as everolimus (also known as RAD001), as a targeted therapy for OC are currently being actively investigated in Phase 1, 2 and 3 clinical trials (34-36).

Metformin is an anti-diabetic medication from the biguanide class that is widely used as the first line treatment of type 2 diabetes. Mounting epidemiological evidence suggests that metformin use lowers cancer risk and reduces cancer deaths among diabetic patients (37-39), including OC (40-42). Metformin may have both indirect and direct effects on tumor growth(43). Its indirect effects are postulated to be due to a reduction in circulating glucose and insulin levels in the host *via* inhibition of gluconeogenesis in the liver, and subsequent decreased growth factor stimulation in tumor cells. On the cellular - or direct - level, metformin inhibits mitochondrial respiratory complex 1, leading to suppression of tricarboxylic acid (TCA) cycle flux, interrupted oxidative phosphorylation and decreased mitochondrial ATP production (43-46). Tumors engineered to express a surrogate for complex 1 that is refractory to metformin were found to be resistant to metformin *in vivo* (46), supporting that metformin's effects on mitochondrial metabolism are critical to direct
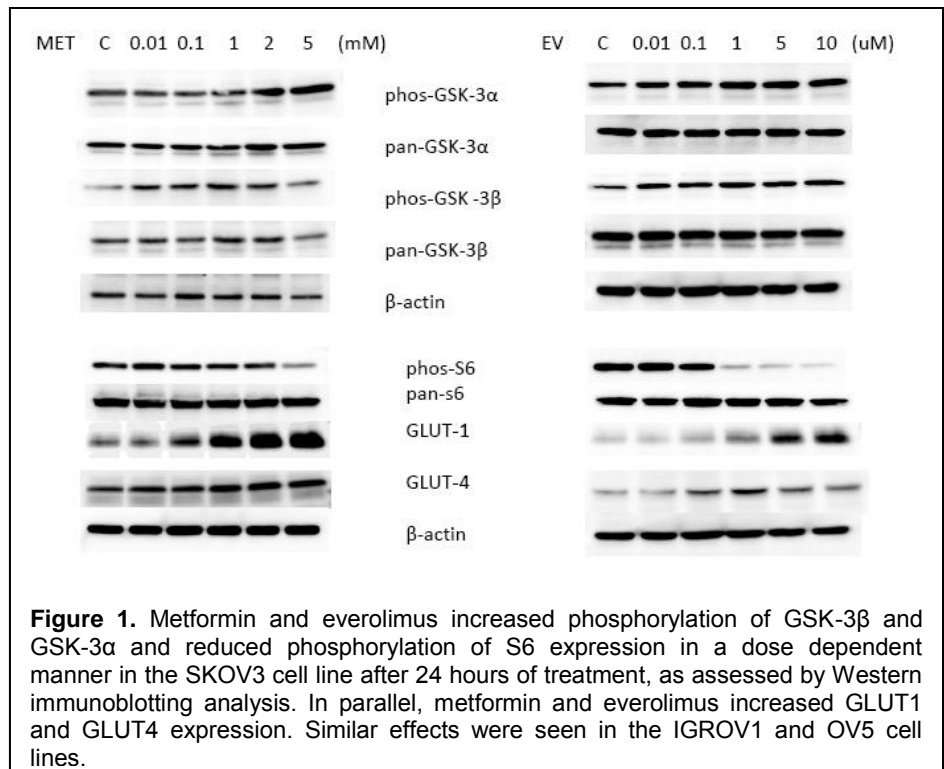
inhibition of tumor growth. The resulting cellular energetic stress from inhibition of complex 1 raises the AMP/ATP ratio, resulting in increased AMPK signaling and stimulated glycolysis and fatty acid oxidation. AMPK is a central regulator of multiple signaling pathways that control cellular proliferation and metabolism, including inhibition of the mTOR pathway (i.e. specifically mTORC1 inhibition) (43). In addition, metformin has also been found to inhibit the mTOR pathway *via* AMPK-independent mechanisms, potentially through its effects on the Ragulator complex (Rag GTPase) and REDD1 upregulation or *via* enhanced PRAS40 binding to RAPTOR (43, 47-50). *Thus, a drug such as metformin that indirectly decreases circulating glucose and insulin levels, inhibits mitochondrial complex 1 and disrupts the mTOR pathway may be useful in an obesity- and mTOR pathway-driven cancer, such as OC.*

Given that we have previously shown that metformin and mTOR inhibitors are potent inhibitors of OC cell proliferation (51, 52), we wanted to assess whether these agents also had an effect on glucose metabolism in OC cell lines. Western immunoblotting analysis revealed that treatment with metformin and the mTOR inhibitor, everolimus, increased facilitative glucose transporter 1 and 4 (GLUT-1 and GLUT-4) expression and increased phosphorylation of glycogen synthase kinase-3 alpha and beta (GSK-α and GSK-β) (**Figure 1**). In parallel, metformin and everolimus
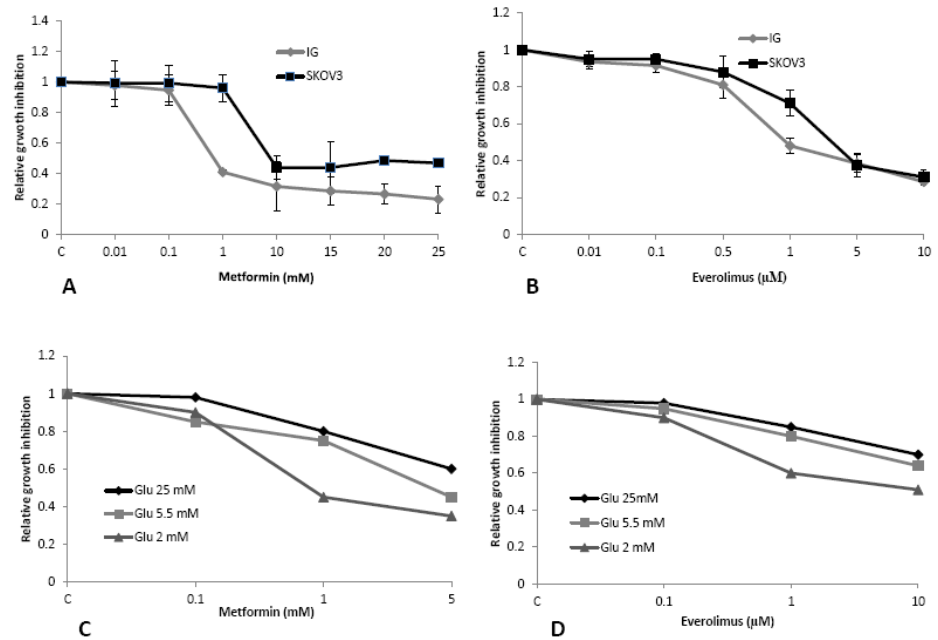

Figure 2. The SKOV3 and IGROV1 (IG) cell lines were treated with metformin and everolimus under low glucose (2 mM), normal glucose (5.5 mM) or high glucose (25 mM) conditions for 72 hours. Cell growth was determined by MTT assay. Metformin and everolimus inhibited proliferation in both cell lines under normal glucose conditions (**A and B**). Metformin and everolimus were found to be more effective in the inhibition of proliferation under low *versus* high glucose concentrations (**C and D**). Similar results were also found for the OV5 cell line.
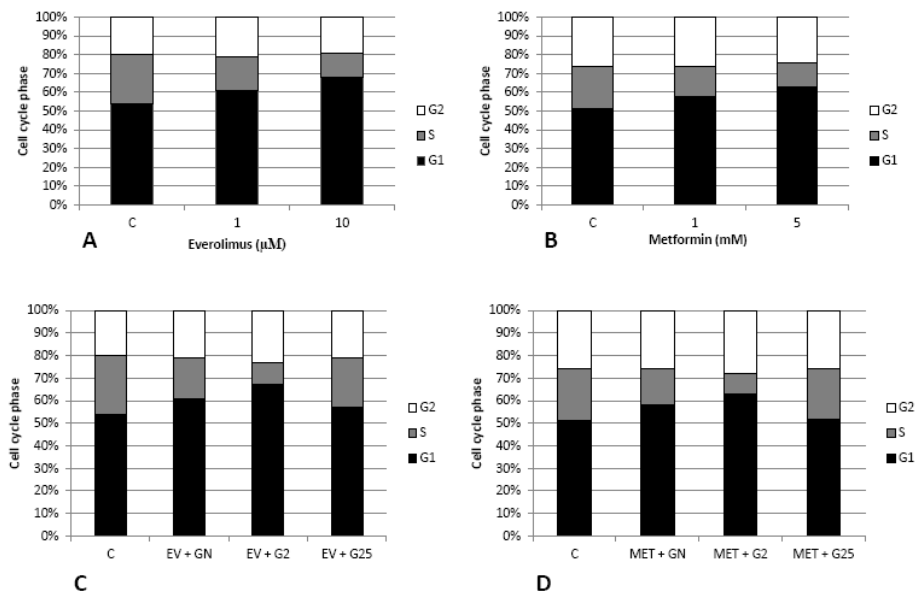

Figure 3. Metformin and everolimus induced G1 cell cycle arrest in the SKOV3 cell line (**A and B**). The SKOV3 cell line was treated with metformin (1 mM) or everolimus (1 uM) under low glucose (2 mM, G2), normal glucose (5.5 mM, GN) or high glucose (25 mM, G25) conditions for 24 hours (**C and D**). Metformin and everolimus were found to be more effective in G1 cell cycle arrest under low *versus* high glucose conditions. Cell cycle phase analysis was assayed by cellometer. Similar results were also found with the IGROV1 and OV5 cell lines. *EV=everolimus, MET=metformin

inhibited the mTOR pathway, as evidenced by decreased phosphorylation of its downstream target, S6 (**Figure 1**). These results suggest that treatment with metformin and everolimus potentially drives glucose metabolism and uptake in OC cells, despite blunting proliferation.

Subsequently, the effects of metformin and everolimus treatment on cell proliferation and apoptosis was assessed under normal, low and high glucose conditions in OC cell lines. Our goal was to mimic the obese-diabetic state *in vitro* through the use of high physiologic glucose. As expected, metformin and everolimus inhibited proliferation in both cell lines under normal glucose conditions (**Figure 2A and 2B**), through G1 cell cycle arrest (**Figure 3A and 3B**). Metformin and everolimus were found to be more effective in the inhibition of proliferation under low *versus* high glucose concentrations (**Figure 2C and 2D**), as also evidenced by enhanced G1 cell cycle arrest (**Figure 3C and 3D**). In addition, metformin and everolimus were found to be more

Figure 4. The SKOV3 cell line was treated with metformin (1 mM; B) or everolimus (1uM; A) under low glucose (2 mM; G2), normal (5.5 mM; GN) or high glucose conditions (25 mM; G25) for 24 hours. Metformin and everolimus were found to be more effective in the induction of apoptosis under low *versus* high glucose concentrations. Annexin V expression, a marker of apoptosis, was assessed by cellometer. Similar results were also found for the IGROV1 and OV5 cell lines. *EV=everolimus. MET=Metformin

effective in the induction of apoptosis under low *versus* high glucose concentrations (**Figure 4**). For all of these experiments, the effects of metformin and everolimus under normal glucose conditions was intermediary between low and high glucose levels. These results were opposite to what we had predicted; we had hypothesized that metformin and everolimus would be more effective under high glucose as opposed to low and normal glucose conditions.

Given these unexpected findings, we did examine the effects of differing glucose conditions (i.e. low, normal and high) alone on AMPK activation and mTOR pathway inhibition in the OC cells. As the concentration of glucose increased, we found that phosphorylation of AMPK decreased and phosphorylation of S6 increased, suggesting increased proliferative capacity and hyperactivation of the mTOR pathway with high physiologic glucose (**Figure 5**). Metformin and everolimus were effective under all glucose conditions in the OC cells; however, we observed heightened sensitivity under low *versus* high glucose conditions. Thus, we postulate that high glucose levels may override some of the anti-proliferative effects of both of these agents in vitro, and that cells exposed to low glucose may have blunted proliferative capacity and may be more inherently susceptible to metformin and everolimus.

The effect of metformin *versus* everolimus on glycolysis and glucose uptake was assessed in the OC cell lines (**Figure 6**). Glucose increased ATP production in the OC cells which was then reversed by treatment with either metformin or everolimus. As expected, metformin increased glucose uptake and everolimus decreased glucose uptake in the OC cell lines. ***Thus, metformin and everolimus both decrease proliferation and glycolysis in OC cells but have opposite effects on glucose***
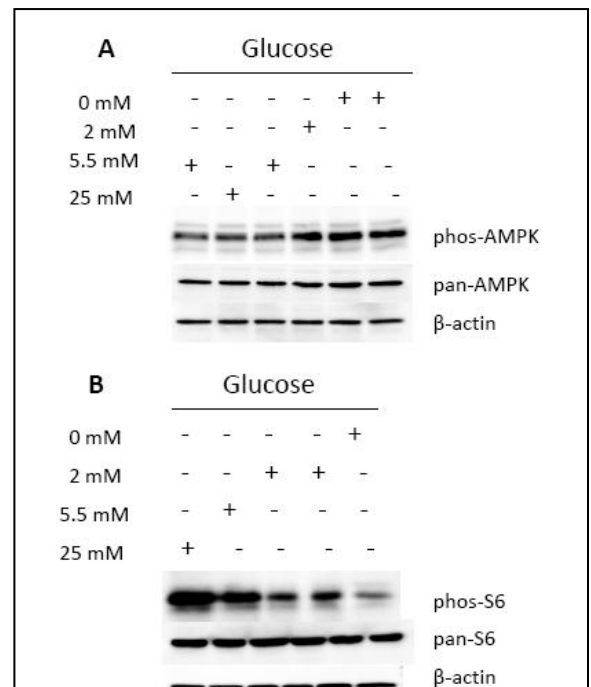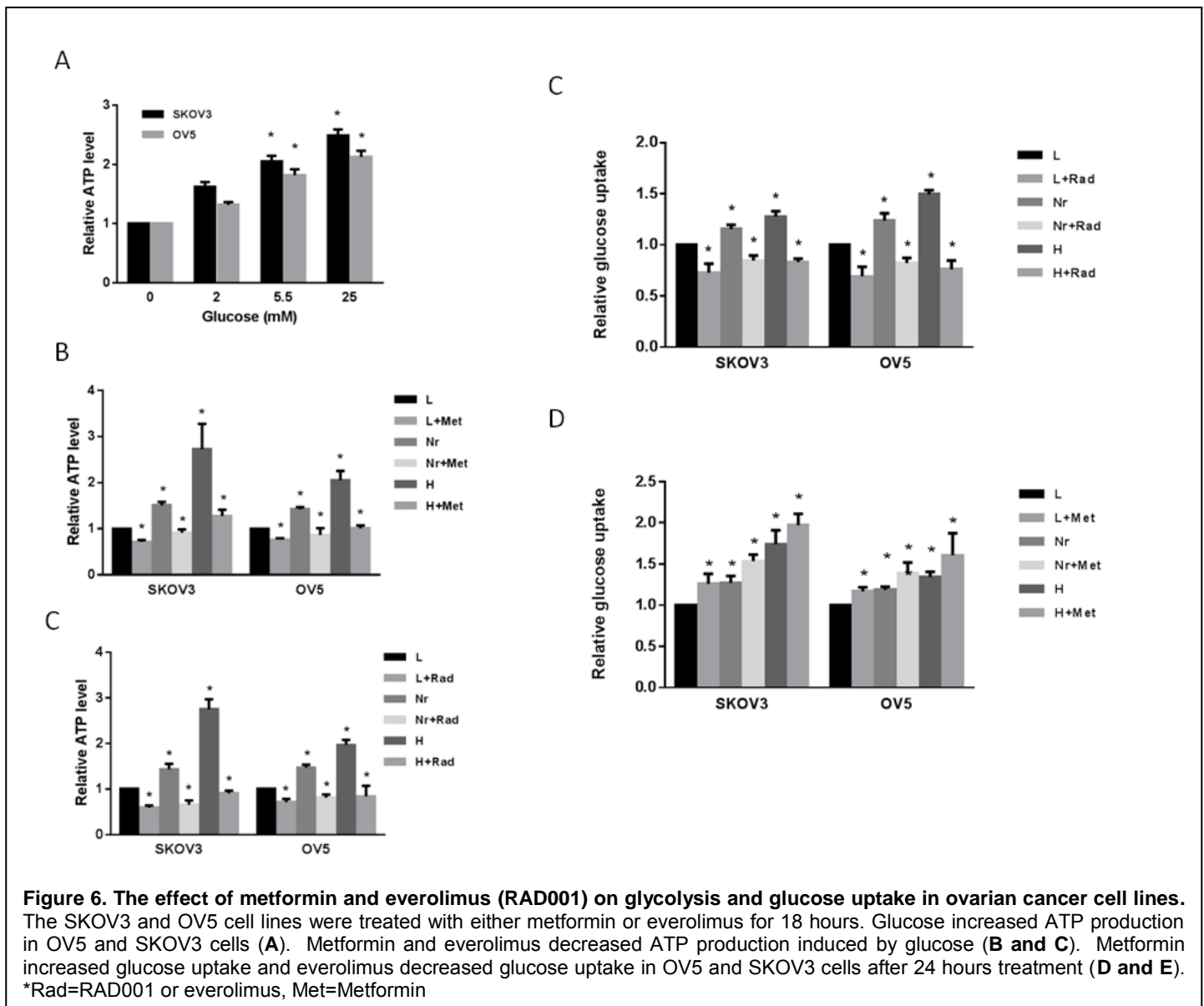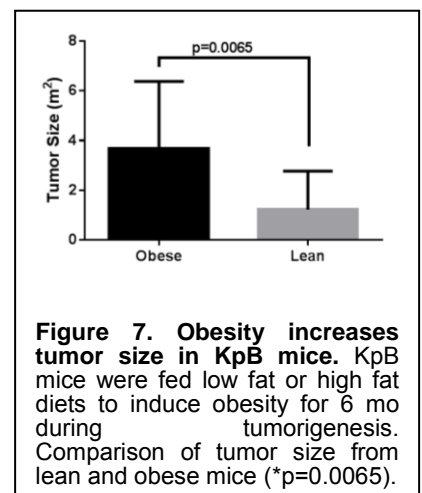

Figure 5. The SKOV3 cell line was exposed to different glucose concentrations for 24 hours. Phosphorylated (phos)-AMPK and S6 and pan-AMPK and S6 expression was determined by Western immunoblotting analysis. As the concentration of glucose increased, phosphorylation of AMPK decreased and phosphorylation of S6 increased. Similar results were found for the IGROV1 and OV5 cell lines.

uptake.

Figure 6. The effect of metformin and everolimus (RAD001) on glycolysis and glucose uptake in ovarian cancer cell lines. The SKOV3 and OV5 cell lines were treated with either metformin or everolimus for 18 hours. Glucose increased ATP production in OV5 and SKOV3 cells (**A**). Metformin and everolimus decreased ATP production induced by glucose (**B and C**). Metformin increased glucose uptake and everolimus decreased glucose uptake in OV5 and SKOV3 cells after 24 hours treatment (**D and E**). *Rad=RAD001 or everolimus, Met=Metformin

Task 2 (Aim 2): To compare tumor latency, growth and response to treatment with the mTOR inhibitor everolimus and metformin in lean and obese K18-gT$_{121}$$^{+/-}$;p53$^{fl/fl}$;Brca1$^{fl/fl}$ (KpB) mice using genomics and metabolomics to identify pathways and biomarkers associated with therapeutic success.

Obesity drives significant OC progression in KpB mice. We have previously assessed the impact of obesity on OC development in a unique serous OC mouse model (K18-gT$_{121}$$^{+/-}$;p53$^{fl/fl}$;Brca1$^{fl/fl}$, here the KpB mouse model). The KpB mouse has specific and somatic deletions of the tumor suppressor genes, *BRCA1* and *TP53*, and inactivation of the retinoblastoma (Rb) protein in adult ovarian surface epithelial cells (53, 54). Inactivation of all 3 Rb proteins by T$_{121}$ (a fragment of the SV40 large T antigen) is driven by the keratin 18 (K18) promoter. Expression of the T$_{121}$ transgene and knockout of p53 and Brca1 are conditional and only activated *via* injection of an

Figure 7. Obesity increases tumor size in KpB mice. KpB mice were fed low fat or high fat diets to induce obesity for 6 mo during tumorigenesis. Comparison of tumor size from lean and obese mice (*p=0.0065).

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adenoviral vector expressing Cre (AdCre) into the ovarian bursa cavity of adult female mice. Within 3 months (mo) post induction, ovarian carcinoma *in situ* is present within the injected ovary. At approximately 6 mo after AdCre injection, tumors develop in the affected ovary, while the un-injected ovary remains normal. As reported through The Cancer Genome Atlas (TCGA) project, RB1, p53 and BRCA1 are central to the pathogenesis of serous OCs in patients (55), which further supports the relevance of our model.

KpB mice were fed a high fat diet (HFD; 60% calories derived from fat) *versus* a control low fat diet (LFD; 10% calories from fat) starting at 6 wks of age to mimic diet-induced obesity (56). AdCre was injected at 8 wks to induce invasive OC. There was a significant difference in body weight between the two groups (obese mice: 50.7 g ± 16.7, lean mice: 31.1 g ± 5.3; p=0.0003)(56). After 6 mo of exposure to HFD/LFD, tumors in HFD-fed (obese-OCs) mice were more than triple the size of those in LFD-fed (lean-OCs) mice (mean tumor size 3.7 cm^2 *versus* 1.2 cm^2, p=0.0065) (**Figure 7**) (56). *Thus, the obese state promotes tumor aggressiveness in the KpB mouse model of serous OC.*
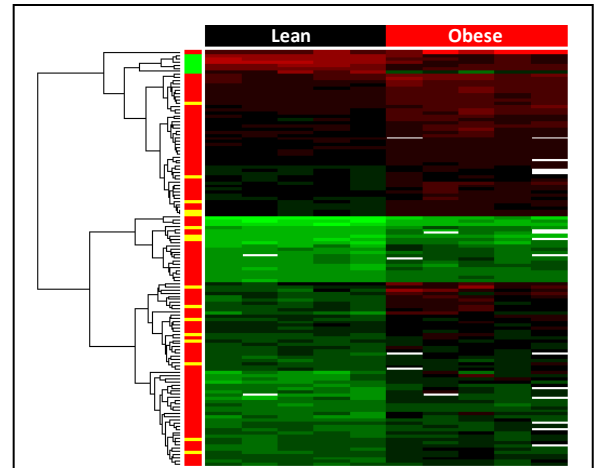

Figure 8. Genomic differences between ovarian tumors from obese *versus* lean KpB mice reveal alterations in metabolically relevant genes. Heat map representation of 131 genes significantly up- or down-regulated in ovarian tumors from obese *versus* lean KpB mice (FDR<0.1).

Genomic and metabolic differences characterize ovarian tumors arising in obese and lean mice. Gene expression and metabolomic profiling indicated statistically significant differences between ovarian tumors arising in obese *versus* lean mice (**Figure 8 & Table 1**)(56). 417 genes were up-regulated and 22 genes down-regulated in ovarian tumors from obese KpB mice *versus* lean mice (FDR<0.2), including genes involved in glucose, fatty acid and lipid metabolism as well as regulators of metabolic signaling pathways such as 5' adenosine monophosphate-activated protein kinase (AMPK). *Thus, the aggressive phenotype of OC in obese KpB mice was accompanied by upregulation of genes involved in metabolic and cell signaling pathways.*

Similarly, metabolomic profiling revealed metabolic differences between ovarian tumors from HFD-fed (obese) and LFD-fed (lean) KpB mice. 58 up- or down-regulated metabolites differentiated ovarian tumors in obese and lean mice. Glutamine (1.7 fold) and several fatty acids metabolites (5-10 fold) were found to be increased in the tumors from obese mice (p<0.05, **Table 1**). Most strikingly, glucose levels were 3-fold higher in the ovarian tumors of the obese *versus* lean mice (p<0.05), and were accompanied by decreases in downstream intermediates of glycolysis, including pyruvate and lactate, indicating impaired glycolysis (**Table 1**). This finding was unexpected because malignant cells generally increase glycolysis, preferentially metabolizing glucose to lactate for ATP production (the "Warburg" effect). As opposed to glucose, the more rapidly growing obese-OCs appeared to incompletely oxidize fatty acids for ATP production and fueling growth as opposed to glucose as evidenced by a 3-4 fold increase in several acyl-carnitines and dicarboxylic acids. Our gene expression profiling data support such a hypothesis as several lipases involved in lipid metabolism are upregulated in obese-*versus* lean-OCs; in addition, the AMPK gene was upregulated, and AMPK is involved in the activation of pathways that generate ATP such as fatty acid oxidation (57, 58) Lastly, succinate levels were almost 5-fold higher in the ovarian tumors from obese *versus* lean mice with a parallel decrease in fumarate and malate, indicating impaired succinate

Sub-pathway	Biochemical Name	Obese/Lean
Glycolysis, Gluconeogenesis and Pyruvate Metabolism	Glucose	2.76
	Fructose-6-phosphate	0.52
	Isobar: F1, 6BP, G1, 6BP, myo-INS BPs	0.45
	Pyruvate	0.48
	Lactate	0.76
TCA cycle	Succinate	4.84
	Fumarate	0.62
	Malate	0.71
Fatty Acid Oxidation	Palmitoylcarnitine	3.22
	Stearoylcarnitine	4.41
	Oleoylcarnitine	4.45
	Azelate	4.55
	Undecanedioate	4.48
Amino Acids	Aspartate	0.81
	Asparagine	0.73
	Glutamate	0.85

Table 1. Comparison of differences in glucose metabolism between the ovarian tumors from obese and lean KpB mice.

dehydrogenase (complex 2) activity. Aspartate, asparagine and glutamate feed into the tricarboxylic acid (TCA) cycle, and these were decreased in obese-OCs, further supporting a block at complex 2. Mitochondrial dysfunction has been reported in tumors cells (59), including that of complex 2 in OC (60). *In summary, our findings suggest that obesity promotes changes in tumor genomics and metabolomics (i.e. an "obesity signature") that lead to aggressive tumor behavior in the KpB OC mouse model.* Once these genomic and

metabolic changes are established, it is unknown if they can be undone by improving the metabolic environment of the host. We hypothesize that the <u>*"obesity-driven signature"*</u> of obese OC tumors is ultimately <u>fixed</u> and thus not responsive to changes in the host metabolic milieu. As part of our future work (Aim 1 of R01 to be resubmitted in 2016), we will answer this question through transplantation of ovarian tumors from both obese and lean mice into lean hosts to track tumor genotype/phenotype.

Figure 9. Metformin inhibited ovarian tumor volume in obese and lean KpB mice. In obese mice, metformin decreased tumor volume by 60% compared to obese control animals. Tumor volume was only decreased by 32% in the lean mice compared to lean controls. A comparison of the anti-tumorigenic effects of metformin on ovarian tumors from lean and obese mice demonstrated that metformin was more efficacious in obese mice (p=0.003), suggesting that metformin may be more beneficial in the setting of obesity.

Metformin has anti-OC effects *in vitro* and *in vivo*: Metformin has been found to inhibit proliferation, migration, angiogenesis, invasion and adhesion in human OC cell lines and mouse models (51, 61-66). Metformin has been shown to have anti-proliferative effects in OC cell lines (67), and to behave synergistically when used in combination with carboplatin and cisplatin in OC cell lines, primary isolates from OC patients and mouse models (65, 68-70).

Importantly, metformin has increased anti-tumorigenic efficacy in our obese KpB mouse model. KpB mice were subjected to a HFD or LFD diet starting at 6 wks of age, and AdCre injection was performed at 8 wks of age. Once tumor growth was confirmed by palpation of a 1 cm tumor, obese and lean mice were treated with vehicle or metformin orally (200 mg/kg/day) for 4 wks (N=10 mice/group). Metformin inhibited tumor volume growth in both the HFD-fed and LFD-fed mice after 4 wks of treatment (**Figure 9**). However, metformin- induced decreases in tumor volume in HFD-fed animals were significantly greater than in LFD-fed animals (60% *versus* 32%, respectively, p=0.003). *Thus, our pre-clinical studies suggest that metformin's anti-tumorigenic efficacy may be dependent on obese and insulin resistant states, which has also been shown in breast and lung cancer mouse models (71-74).*

Immunohistochemical analysis was performed of the ovarian tumors after treatment with metformin or vehicle to assess effects on proliferation, apoptosis and downstream targets of the mTOR pathway (**Figure 10**). As compared to vehicle-treated mice, metformin decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors of obese and lean KpB mice. In addition, metformin increased phosphorylation of AMPK (activating it) and decreased phosphorylation of S6, a downstream target of the mTOR pathway.
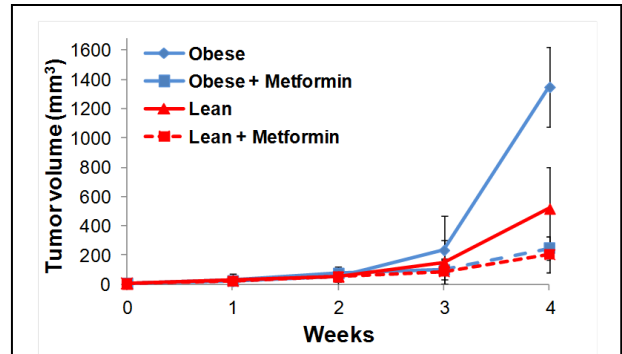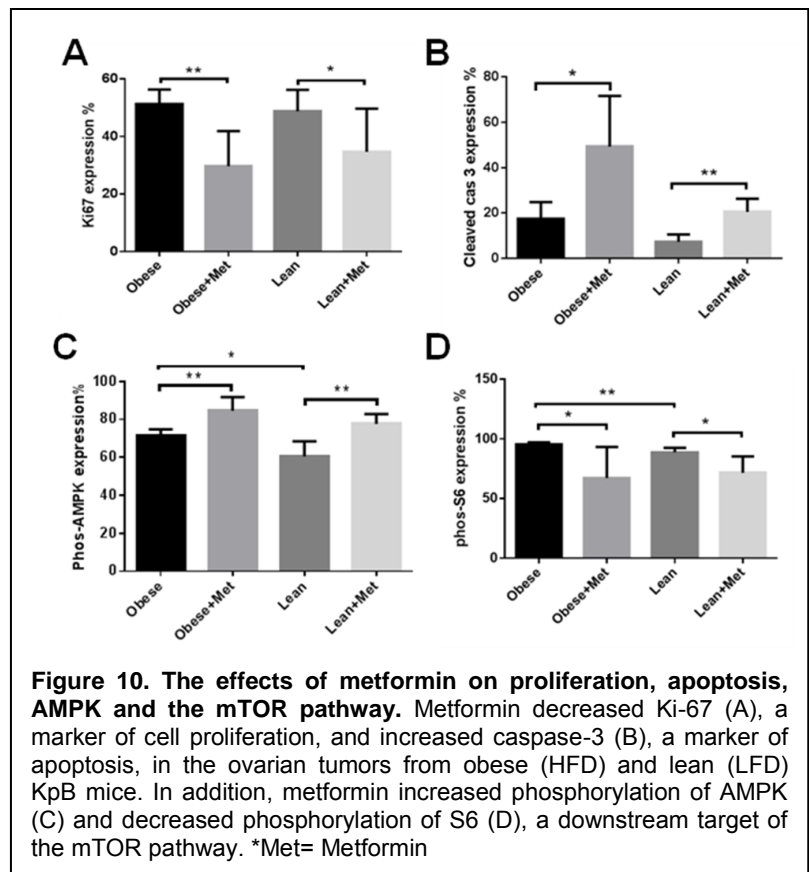
Metabolomic profiling was performed on

Figure 10. The effects of metformin on proliferation, apoptosis, AMPK and the mTOR pathway. Metformin decreased Ki-67 (A), a marker of cell proliferation, and increased caspase-3 (B), a marker of apoptosis, in the ovarian tumors from obese (HFD) and lean (LFD) KpB mice. In addition, metformin increased phosphorylation of AMPK (C) and decreased phosphorylation of S6 (D), a downstream target of the mTOR pathway. *Met= Metformin

ovarian tumors from the obese and lean KpB mice treated with either vehicle or metformin. Glucose levels were initially high within ovarian tumors of obese mice, falling with metformin treatment. Glycolysis was preferentially stimulated in the ovarian tumors of obese mice treated with metformin as compared to lean mice, suggesting a switch in substrate from fatty acids to glucose, as evidenced by elevations in glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, dihydroxyacetone phosphate and most importantly, lactate ($p < 0.05$, **Table 2**).

Metformin is known to decrease mitochondrial respiration efficiency by inhibiting mitochondrial complex 1 and thus shifting the ATP production burden to anaerobic glycolysis; succinate was relatively depleted whereas fumarate and malate appeared to accumulate in metformin-treated tumors – *but only in obese mice* – a result consistent with restricted conversion of malate to oxaloacetate (complex 1 activity) ($p < 0.05$, **Table 2**). These metabolic changes may underlie why obese-OCs have heightened susceptibility to metformin, i.e., obese-OCs have impaired mitochondrial complex 2 function that when combined with metformin's inhibition of complex 1 leads to profound impairment of mitochondrial oxidative phosphorylation (**Figure 11**). Thus, the obese-OCs may become solely dependent on glycolysis for ATP production. Further supportive evidence is the dramatic rise in n3 and n6 fatty acids with metformin treatment in obese-OCs, indicating an inability to oxidize fatty acids when mitochondrial complex 1 and 2 are inhibited (**Table 2**).

Sub-Pathway	Biochemical Name	Lean-Met/ Lean-Ctrl	Obese-Met/ Obese-Ctrl
Glycolysis	Glucose	2.75	0.46
	Glucose-6-phosphate	0.91	1.82
	Fructose-6-phosphate	0.87	2.3
	Fructose-1-6-bisphosphate	0.68	2.4
	Dihydroxyacetone phosphate	0.64	2.44
	Lactate	0.82	1.35
TCA Cycle	Citrate	0.46	0.94
	Alpha-ketoglutarate	1.23	1.53
	Succinylcarnitine	3.6	2.44
	Succinate	3.29	0.22
	Fumarate	0.73	1.57
	Malate	0.8	1.4
Glutamine Oxidation	α-ketoglutarate	1.23	1.53
	Glucosamine-6-phosphate	0.65	1.8
	N-acetylglucosamine-6-phosphate	0.56	2.13
n3 and n6 Fatty Acids	Eicosapentaenoate	0.54	1.12
	Docosapentaenoate	0.48	1.94
	Docosahexaenoate	0.69	1.97
	Dihomo-linolenate	0.99	2.23
	Arachidonate	0.90	2.28
	Adrenate	0.51	2.1
	Docosapentaenoate	0.72	3.75
	Docosadienoate	0.57	1.83

Table 2. Comparison of metabolic changes with metformin treatment in the ovarian tumors from obese and lean KpB mice**.**

Glutamate can also be oxidized to generate TCA cycle intermediates; this process was induced in the metformin-treated obese-OCs, possibly as a mechanism to overcome metformin's inhibitory effects on mitochondrial metabolism (**Table 2**). *These findings suggest that metformin may have differential <u>direct</u> metabolic effects to alter the established metabolic phenotype of ovarian tumors in obese versus lean mice, leading to improved efficacy in treating tumors which develop in an obese host environment.* As demonstrated in **Table 1**, obese-OCs appear more reliant on fatty acid oxidation as opposed to glycolysis for ATP production with coincident impaired mitochondrial complex 2 function. In contrast, the opposite was true for lean-OCs. *Given that the effects of metformin to inhibit the mTOR pathway were similar between ovarian tumors from obese and lean mice (Figure 10), we postulate that metformin's direct metabolic effects on inhibition of mitochondrial complex 1 drive the increased response in complex 2 impaired tumors from obese mice (Figure 11).* The manuscript of the effects of metformin in obese and lean KpB mice will be submitted in December 2015.
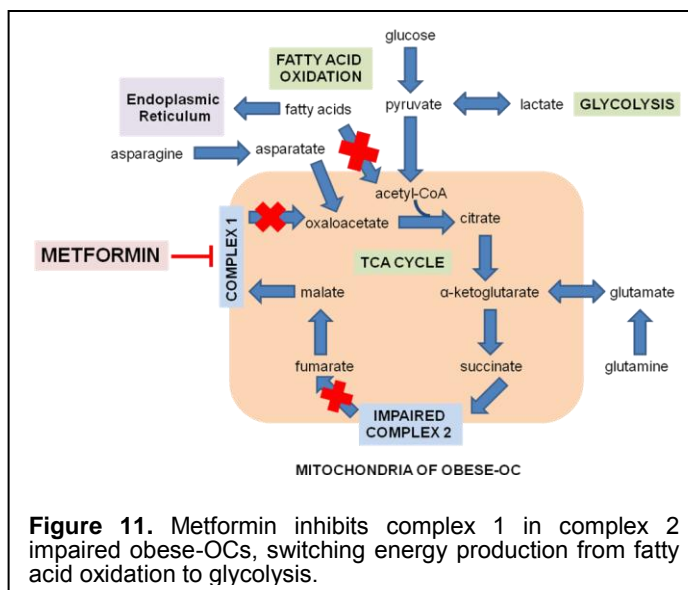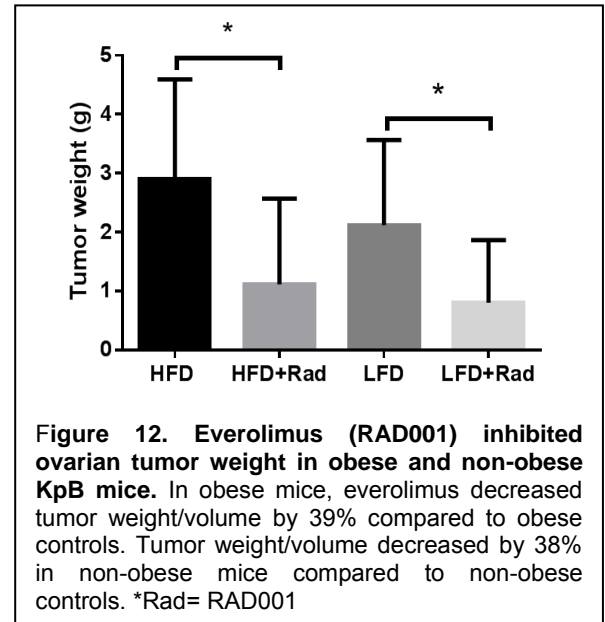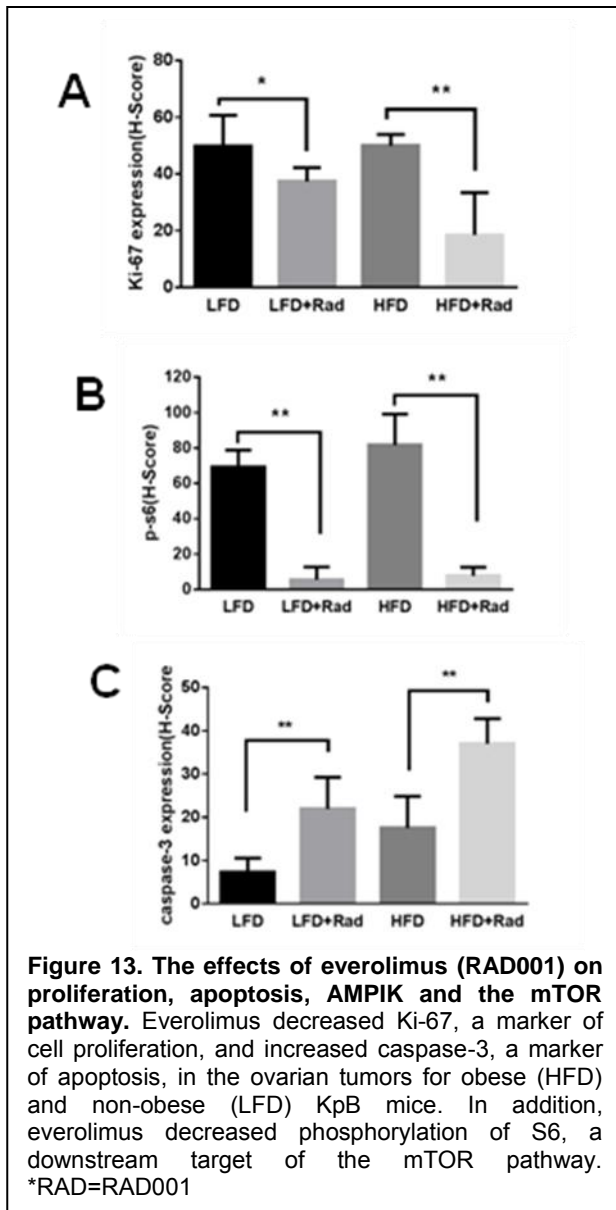

Figure 11. Metformin inhibits complex 1 in complex 2 impaired obese-OCs, switching energy production from fatty acid oxidation to glycolysis.

Everolimus (RAD001) has anti-OC effects *in vitro* and *in vivo*: Everolimus (3 mg/kg/day, intraperitoneally) inhibited tumor growth in the KpB mice fed a LFD and a HFD (n=11-13 animals per group), after one month of treatment (**Figure 12**). In the mice fed a HFD, everolimus decreased tumor weight and volume by 39% compared to control animals. Tumor weight and volume was decreased by 38% in the mice fed a LFD. Thus, in contrast to metformin, everolimus had equivalent effects on the inhibition of ovarian tumor growth in the obese (HFD) and non-obese (LFD) KpB mice.

Immunohistochemical analysis was performed of the ovarian tumors after treatment with everolimus or control to assess for effects on proliferation, apoptosis and downstream targets of the mTOR pathway (**Figure 13**). As compared to control-treated mice, everolimus decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors for obese (HFD) and non-obese (LFD) KpB mice. In addition, everolimus decreased phosphorylation of S6, a downstream target of the mTOR pathway.

Metabolomic profiling was performed on ovarian tumors from the obese and lean KpB mice treated with either vehicle or everolimus.

Figure 12. Everolimus (RAD001) inhibited ovarian tumor weight in obese and non-obese KpB mice. In obese mice, everolimus decreased tumor weight/volume by 39% compared to obese controls. Tumor weight/volume decreased by 38% in non-obese mice compared to non-obese controls. *Rad= RAD001

Figure 13. The effects of everolimus (RAD001) on proliferation, apoptosis, AMPIK and the mTOR pathway. Everolimus decreased Ki-67, a marker of cell proliferation, and increased caspase-3, a marker of apoptosis, in the ovarian tumors for obese (HFD) and non-obese (LFD) KpB mice. In addition, everolimus decreased phosphorylation of S6, a downstream target of the mTOR pathway. *RAD=RAD001

Metabolites involved in polyamine metabolism were down-regulated after treatment with everolimus in both obese and lean KpB mice as compared to control mice, including putrescine, spermidine and N-acetylputrescine ($p < 0.05$) (**Table 3**). These results suggest that everolimus was able to reduce polyamines needed for packaging newly synthesized DNA into chromatin within the nucleus and are indicative of a decrease of cell proliferation in tumor tissues. Increased phosphoenolpyruvate (PEP) and decreased pyruvate levels in the glycolytic pathway were observed in obese and lean everolimus-treated mice, suggesting everolimus inhibited glycolysis through inhibition of pyruvate kinase (PK) (**Table 3**), although glucose levels were 3 fold higher in the tumors of obese *versus* lean mice ($p < 0.05$) (**Table 1**). Lower levels of gut microbiome-associated metabolites and compounds absorbed from the diet: pipecolate, hippurate, catechol sulfate, stachydrine, ergothioneine and indolepropionate, were detected with everolimus treated obese and lean mice ($p < 0.05$) (**Table 3**); and thus, mTOR suppression may have systemic effects through alteration of the composition of circulating nutrients in tumor tissues. The glycerophosphodiesters, glycerophosphorylcholine (GPC), glycerophosohoethanolamine (GPE) and glycerophosphoglycerol (GPG), were found to be significantly lower in obese and lean mice treated with everolimus ($p < 0.05$) (**Table 3**), reflecting that everolimus may activate glycerophosphodiesterase(s) in tumor tissues. Significant reductions of dipeptides were found in obese but not lean mice treated with everolimus, suggesting everolimus

decreases protein degradation to a greater extent in obese mice (**Table 4**). Additionally, significant elevations of intermediates of pyrimidine metabolism were found in lean control mice, which decreased significantly after everolimus treatment in lean but not obese mice (**Table 5**). *These metabolomic studies support that everolimus has differential actions depending on whether the ovarian tumors arise from obese or lean mice.* The manuscript of this work on everolimus in OC cell lines and the KpB mouse model has been submitted to *Oncotarget*.

RNA has been extracted from these tumors post-metformin and post-everolimus treatment and is undergoing genomic analysis. Serum has been collected from these mice pre- and post-treatment with metformin and everolimus for (1) measurement of glucose, leptin, insulin, IGF-1 and adiponectin, and (2) metabolomic analysis.

Sub-Pathway	Biochemical Name	LFD-Rad/ LFD-control	HFD-Rad/ HFD-control
Polyamine Metabolism	Putrescine	0.11	0.16
	Spermidine	0.09	0.42
	N-acetylputrescine	0.11	0.07
Glycolysis	Phosphoenolpyruvate	3.57	1.95
	Pyruvate	0.27	0.42
Gut Microbiome	Pipecolate	0.29	0.36
	Hippurate	0.15	0.29
	Catechol Sulfate	0.05	0.12
	Stachydrine	0.2	0.29
	Ergothioneine	0.04	0.09
	Indolepropionate	0.17	0.15
Glycerophosphodiesters	Glycerophosphorylcholine	0.11	0.07
	Glycerophosphoethanolamine	0.16	0.12
	Glycerophosphoglycerol	0.19	0.19

Table 3. Metabolic changes with everolimus treatment in the ovarian tumors from lean (LFD-fed) and obese (HFD-fed) mice. (p<0.05)

Task 3 (Aim 3): Cross-species evaluation of differences between human and KpB mouse ovarian cancers in lean and obese states through genomics and metabolomics and primary culture.

We have collected ovarian tumors for primary culture from 10 OC patients to date. The tumors from these 10 patients were treated with metformin and everolimus in short term primary culture. Metformin (IC50 range 1-5 mM) and everolimus (IC50 range 68-250 nM) inhibited cell proliferation in all of the primary cultures to date, with parallel decreases in phosphorylation of S6, a downstream target of the mTOR pathway. We have not found any significant differences to response to metformin or everolimus in the ovarian tumors from obese *versus* non-obese women. However, this is most likely related to the limitations of *in vitro* culture, where it is difficult to replicate the *in vivo* metabolic environment of obesity as we have done in Task 2.

Ovarian tumors from obese and non-obese OC patients have been identified in the LCCC tissue bank and are being sent to Metabolon for metabolomic analysis. The results from this analysis will be compared to that of the metabolomic analysis of ovarian tumors from obese and non-obese KpB mice.

To assess genomic differences between high grade serous ovarian tumors from obese *versus* non-obese women, we took advantage of the publically available gene expression analysis from The Cancer Genome Atlas (TCGA) database. From the TCGA database, we collected expression measurements for 12,042 genes from the platform (BI_HT_HG-

Biochemical Name	HFD-Rad/ HFD-control
Alanylleucine	0.06
Glutamine-leucine	0.07
Glycylisoleucine	0.1
Glycylleucine	0.21
Glycylvaline	0.15
Isoleucylglycine	0.19
Lyslleucine	0.07
Phenylalanylalanine	0.03
Phenylalanylglycine	0.07
Proylglycine	0.11
Tryptophylglycine	0.05
Valylglycine	0.1

Table 4. Dipeptides that decreased significantly with everolimus treatment in the ovarian tumors from only obese (HFD-fed) mice. (p<0.05)

Biochemical Name	LFD-Rad/ LFD-control
Uridine-5'-monophosphate	0.01
Uracil	0.44
Psuedouridine	0.53
3-ureidopropionate	0.53
Beta-alanine	0.34
N-acetyl-beta-alanine	0.16

Table 5. Intermediates of pyrimidine metabolism that decreased significantly with everolimus treatment in the ovarian tumors from only lean (LFD-fed) mice. (p<0.05)

U133A level 3 data) for differential gene expression analysis among human high grade, serous OC samples. The detailed information of the data processing, quality control and normalization can be found on the TCGA website. To identify significantly differentially expressed genes associated with BMI, we applied linear modeling for responses as gene expression and covariates as 5 principal components (PCs) (from gene expression data

to control potential batch effects), clinical stage, grade, age, race, residual tumor and BMI status (0 if normal BMI < 25; 1 if overweight BMI ≥ 25). Appropriate false discovery rates (FDR) were controlled. With the obtained genes that were significantly associated with BMI status, we conducted functional clustering analysis on the website of The Database for Annotation, Visualization and Integrated Discovery (DAVID). In addition,

we applied hierarchical clustering analysis to generate a representative heatmap. The Chi-square test was used to compare BMI among different clusters of samples. A comparison of the demographics between the OC tumors from normal weight (BM I< 25) and

	BMI < 25 (Normal Weight) (N=99)	BMI ≥ 25 (Overweight/Obese) (N=138)
Age (mean)	57.9	59.4
Race		
White	89 (90%)	125 (91%)
Black	5 (5%)	11 (8%)
Other	5 (5%)	2 (1%)
Grade		
2	11 (11%)	12 (9%)
3	88 (89%)	126 (91%)
Stage		
I/II	2 (2%)	4 (3%)
III/IV	97 (98%)	134 (97%)
Residual Disease		
Optimal	75 (76%)	99 (72%)
Suboptimal	24 (24%)	39 (28%)

Table 6. Comparison of the demographics between the ovarian cancer tumors from normal weight and overweight/obese women.

overweight/obese women (BMI ≥ 25) can be found in **Table 6**.

347 genes were found to be significantly up- or down-regulated with BMI status (BMI < 25 *versus* BMI ≥ 25) among the serous ovarian tumors (q-value < 0.1), including metabolically relevant genes. Genes that were down-regulated included the prolactin receptor (3.6 fold) and apolipoprotein B mRNA editing enzyme (3.1 fold), among others. Genes that were up-regulated included mitogen-activated protein kinase 1 (3.3 fold), phospholipid scramblase 1 (3.3 fold), carnitine/acylcarnitine translocase (3.2 fold), low density lipoprotein receptor-related protein 8 (apolipoprotein e receptor) (3.7 fold), apolipoprotein L3 (3.7 fold), apolipoprotein L1 (3.8 fold), lipoyltransferase 1 (4.2 fold), apolipoprotein L6 (4.2 fold) and the c-myc binding protein (4.1 fold). Many of these genes were related to the apolipoprotein pathway, particularly apolipoprotein L related genes. Apolipoprotein L genes are members of the high density lipoprotein family and play a central role in cholesterol transport. Multiple genes involving the Ras oncogene family were up- and down-regulated when comparing normal weight *versus* overweight/obese women, including ras responsive element binding protein 1, RAB5C, RREB1, ras-related GTP binding C, PAP1A, RAB7A, RAB31, RAB5A, and ras homolog family/member A. DAVID functional annotation analysis revealed significant enrichment in "protein transport" (Adjusted p-value for Benjamini = 5.5E-5), "antigen processing and presentation of exogenous peptide antigen" (Adjusted p-value for Benjamini = 1.3E-3) and "pyrimidine ribonucleotide biosynthetic process" (Adjusted p-value for Benjamini = 3.6E-2) for these identified genes.

Initially, we used the 347 genes with q-value < 0.1 to generate a heatmap, but the results of the hierarchical cluster analysis on these samples did not group them with a significantly different BMI distribution. Alternatively, we used the 175 genes with q-value < 0.05 to generate a heatmap, which is presented in **Figure 14**, where the row signifies gene expression and the column is clustering according to BMI (BMI < 25 *versus* BMI ≥ 25). If we specified two groups to cut a tree resulting from the
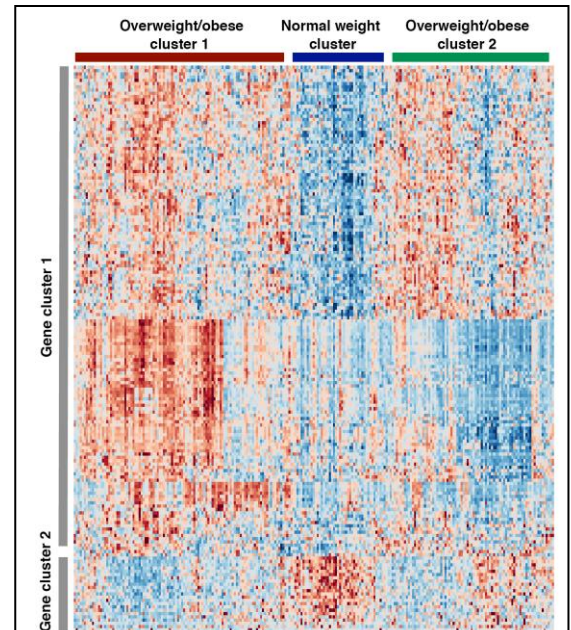

Figure 4. Genomic differences between ovarian tumors from normal weight *versus* overweight/obese women reveal alterations in metabolically relevant genes. Heat map representation of 347 genes significantly up- or down-regulated in ovarian tumors from normal weight *versus* overweight/obese women (FDR<0.1). Analysis of this gene set resulted in three sample clusters, with statistically significant differences in proportions of women with BMI ≥ 25 *versus* BMI <25 among these clusters.

results of the hierarchical cluster analysis on the samples, the two clusters of samples had no statistically significant difference in the distribution of BMI. However, if we specified three groups to cut a tree resulting from the results of the hierarchical cluster analysis, there were two pairs of clusters of samples with a significantly different distribution of BMI. Specifically, the first pair of clusters of samples (cluster 1 *versus* cluster 2) had sample proportions of subjects with BMI ≥ 25 (0.65, 0.33). For testing if the two proportions are significantly different, the obtained Chi-square statistics was 7.87, df = 1 and p-value = 0.005, suggesting that the two sample proportions are significantly different. The second pair of clusters of samples (cluster 3 *versus* cluster 2) had sample proportions of subjects with BMI ≥ 25 (0.61, 0.33). For testing if the two proportions are significantly different, the obtained Chi-square statistics was 11.36, df = 1 and p-value = 0.00075, suggesting that the two sample proportions are significantly different. In addition, there was significant difference in the proportions of women with a BMI ≥ 25 for cluster 1 and cluster 3 (0.65, 0.61). In summary, the analysis of the 175 gene set resulted in three sample clusters, with statistically significant differences in proportions of women with BMI ≥ 25 *versus* BMI <25 among these clusters. A summary of the genes in gene cluster 1 and 2 can be found in **Table 7**.

	Gene Name	David Gene Name
Gene Cluster 1	FAP	Fibroblast activation protein, alpha
	LAIR1	Leukocyte-associated immunoglobulin-like receptor 1
	GPR65	G protein-coupled receptor 65
	RAB5C	RAB5C, member RAS oncogene family
	CTSK	Cathepsin K
	RHOA	Ras homolog gene family, member A
	RAB5A	RAB5A, memver RAS oncogene family
	IL10RA	Interleukin 10, alpha
	IL2RB	Interleukin 10, beta
	LRP8	Low density lipoprotein receptor-related protein 8
	APOL3	Apolipoprotein L3
	APOL1	Apolipoprotein L1
	CFLAR	CASP8 and FADD-like apoptosis regulator
	PLAU	Plasminogen activator, urokinase
	RAB31	RAB31, member RAS oncogene family
	MYCBP	c-myc binding protein
	AAPOL6	Apolipoprotein L6
	LIPT1	Lipoyltransferase 1
	PRKAA1	Protein Kinase, AMP-activated, alpha 1 catalytic subunit
	PTPRC	Protein tyrosine phosphotase, receptor, type C
	ETF1	Eukaryotic translational termination factor 1
	EIF2B3	Eukaryotic translational initiation factor 2B
	CASP1	Caspase 1, apoptosis-related cysteine peptidase
Gene Cluster 2	IGSF3	Immunoglobulin superfamily, member 3
	PRLR	Prolactin receptor
	GRM4	Glutamate receptor, metabotropic 4
	LY6G6E	Lymphocyte antigen 6 complex, locus G6E
	GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1
	ADRA1A	aArenergic, alpha-1A-, receptor

Table 7. Gene Clusters of the Ovarian Tumors from Normal Weight (BMI<25) and Overweight/Obese Women (BMI≥25).

Cross-species comparisons between gene expression profiles of ovarian tumors from obese and non-obese women and mice revealed several upregulated genes in common, related to a diverse array of functions (**Table 8**). The relationship between these genes and obesity-driven ovarian cancers will be a focus of our future work. Metabolic profiling of ovarian tumors from obese and non-obese women Is currently ongoing and will be compared to our results in the KpB mouse model.

Gene	Gene Name	Gene Function
NEBL	nebulette	Regulation of length of actin thin filaments in cardiac muscle
TRIM31	tripartite motif-containing 31	Unknown
PADi4	peptidyl arginine deiminase, type IV	Granulocyte and macrophage development
NAPA	N-ethylmaleimide sensitive fusion protein attachment protein alpha	Membrane fusion
A4GNT	alpha-1,4-N-acetylglucosaminyltransferase	Biosynthesis of heparin and heparin sulfate

Table 8. Genes upregulated in ovarian tumors from obese mice and women *versus* non-obese.

(4) KEY RESEARCH ACCOMPLISHMENTS

- Metformin and everolimus potentially drive glucose metabolism and uptake in OC cells, despite blunting proliferation.
- Metformin and everolimus inhibited proliferation in both OC cell lines under normal glucose conditions, through G1 cell cycle arrest.
- Metformin and everolimus were found to be more effective in the inhibition of proliferation under low *versus* high glucose concentrations, as also evidenced by enhanced G1 cell cycle arrest. In addition, metformin and everolimus were found to be more effective in the induction of apoptosis under low *versus* high glucose concentrations.
- As the concentration of glucose increased, phosphorylation of AMPK decreased and phosphorylation of S6 increased, suggesting increased proliferative capacity and hyperactivation of the mTOR pathway with high physiologic glucose.
- Metformin and everolimus both decreased glucose-stimulated ATP production, but had opposite effects on glucose uptake. Metfomin increased glucose uptake, and everolimus decreased glucose uptake.
- The obese state can promote tumor progression in the KpB mouse model of OC.
- Distinct metabolic and genomic differences were identified in ovarian tumors that arose in obese *versus* lean KpB mice, and many of these differences were related to metabolic relevant pathways.
- Diet induced-obesity (DIO) promoted tumor growth in the KpB genetically engineered mouse model of serous OC, coincident with mitochondrial dysfunction and energy supplied by fatty acid oxidation rather than glycolysis in tumors from obese *versus* lean mice.
- Metformin more efficacious in the inhibition of OC tumor growth in obese *versus* lean mice, which corresponded with inhibition of mitochondrial complex 1, halting of fatty acid oxidation and stimulation of glycolysis in <u>only</u> tumors from obese mice.
- Everolimus had similar effects in the inhibition of tumor growth in obese and non-obese KpB mice but did have differential actions depending on whether the ovarian tumors arose from obese or lean mice.
- Metabolically relevant alterations in gene expression were found with increasing BMI among human serous OCs, using the TCGA database.
- Cross-species comparisons between gene expression profiles of ovarian tumors from obese and non-obese women and mice revealed several upregulated genes in common, related to a diverse array of functions

(5) CONCLUSION

Epithelial OC is one of the most lethal cancers among women in the United States, and minimal improvements in overall survival have been made in the past several decades. The lack of progress is largely attributable to late detection, drug resistance, and a high recurrence rate. Although chemotherapeutic agents that target specific cell signaling pathways have greatly expanded our profile of OC treatments, the challenge has been in identifying the patients that would most benefit from each of these diverse agents. To address these challenges, most previous research has focused on molecular alterations in the tumors derived from these patients. We postulate that focusing on the tumor alone may be too narrow a view and that the host environment, particularly the obese state, may play an equally important role in the selection of chemotherapeutic agents for effective treatment response. <u>It is our hypothesis that obesity drives OC formation through alterations in metabolic pathways; and thus, inhibitors of one such pathway (mTOR) may be more efficacious in the obese *versus* non-obese state.</u>

In order to answer this fundamental biological question regarding the role of the obese environment in oncogenesis, our approach is three fold using OC cell lines, a genetically engineered mouse model and patient samples. For each of these strategies, the metabolic state of obesity is being uniquely invoked and to test our hypothesis, two targeted mTOR pathway agents (metformin and everolimus) are being evaluated under obese *versus* non-obese conditions. To date, we have demonstrated that the obese state can promote tumor progression in the KpB mouse model of OC, as evidenced by a tripling of tumor size in obese *versus* non-obese mice. Diet-induced obesity was mimicked in the KpB mice through exposure to a HFD. The ovarian tumors that arose in the obese mice were genomically and metabolically different from those that arose in non-obese mice. In particular, ovarian tumors from obese *versus* lean mice had evidence of mitochondrial dysfunction and energy supplied by fatty acid oxidation as opposed to glycolysis.

Metformin, an AMPK activator, was found to be more efficacious in the inhibition of ovarian tumor growth in the obese *versus* lean KpB mice, suggesting that obesity may be a biomarker for response to this agent. In contrast, everolimus (RAD001), a mTOR inhibitor, was found to be equally efficacious in obese and lean mice. This difference in findings between these two targeted agents may be partially explained in that metformin increases glucose uptake and everolimus decreases glucose uptake as demonstrated in our *in vitro* studies and by others (75). In addition, our metabolomic results of the effects of metformin *versus* everolimus in the ovarian tumors from obese and lean KpB mice indicate that both agents had differential effects depending on obesity status, although these results were much more striking for metformin. Metformin's increased efficacy in obese *versus* lean mice corresponded with inhibition of mitochondrial complex 1, halting of fatty acid oxidation and stimulation of glycolysis in <u>only</u> tumors from obese mice. Everolimus was equally effective in obese *versus* lean mice but was found to decrease protein degradation more in the obese setting and to reduce pyrimidine metabolism more in the lean setting.

For our *in vitro* studies, obesity/diabetes was mimicked by exposing OC cell lines to high *versus* low and normal glucose conditions. We had hypothesized that metformin and everolimus would be more efficacious in the setting of high *versus* low and normal glucose, but we found the opposite to be true. Metformin and everolimus were found to be more effective in the inhibition of proliferation under low *versus* high glucose concentrations, as also evidenced by enhanced G1 cell cycle arrest. In addition, metformin and everolimus were found to be more effective in the induction of apoptosis under low *versus* high glucose concentrations. We postulate that OC cells deprived of glucose may have blunted proliferative capacity, rendering them to be more susceptible to metformin and everolimus. Our studies also indicate that a high glucose environment enhances proliferative capacity. Metformin and everolimus were both found to decrease glycolysis in OC cells but had opposite effects on glucose uptake, with metformin increasing and everolimus decreasing glucose uptake.

We understand that the *in vitro* environment of high glucose exposure may not completely replicate that of the whole body *in vivo*; and thus, our animal and human studies are purposely meant to complement the cell culture work to better define the impact of obesity on OC development, progression and ultimately, treatment. In order to explore the impact of obesity on sensitivity to mTOR inhibitor and metformin therapy in the human disease, primary cultures of freshly isolated human OC cells derived from obese *versus* lean patients have been exposed to these agents. To date, we have not found any differences in response to metformin or everolimus between ovarian tumors from obese *versus* non-obese women in primary culture.

Alterations in gene expression were found with elevated BMI in serous OC tumors in the TCGA database. Many of the genes with differential expression were related to lipid metabolism and the apolipoprotein pathway, which is important in triglyceride and cholesterol transport, and this pathway will be a focus of our future work. Our findings demonstrate that obesity may contribute to OC pathogenesis through the differential expression of metabolically relevant genes. Metabolomic analysis of ovarian tumors from obese and non-obese women is underway, and these results will be correlated to the gene expression profiling results as well as parallel studies in the KpB mouse model.

Findings from this proposal should determine if obesity-driven OCs are biologically divergent and whether these inherent differences play a role in sensitivity to chemotherapeutic agents, and we have already provided evidence to support this hypothesis. This work may ultimately lead to the individualization of OC treatment based on both tumor biology and the metabolic composition of the patient. This study will initially investigate this concept for the mTOR inhibitor everolimus and metformin in OC, but may <u>ultimately translate to other emerging therapies targeted to this pathway or others identified in this proposal</u>. Future clinical trials of targeted therapies would have to be structured to address the host/tumor interaction, and we would propose that stratifying for obesity status may be an initial approach in this pursuit. <u>To our knowledge, no cancer chemotherapeutic clinical trial has addressed obesity status as a contributing factor to therapeutic success</u>. Lastly, this knowledge of the impact of obesity on response to targeted therapies would be important not just for OC but for all cancers where obesity is associated with increased risk and worse outcomes, such as endometrial, breast and colon cancer among others.

(6) PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Abstracts presented:

(1) Zhou, C, Zhong, Y, Du, X, Makowski, L, Jia, W and Bae-Jump, VL, Diet-induced obesity increases tumor aggressiveness in a genetically engineered mouse model of serous ovarian cancer, 44th Annual Meeting of the Society of Gynecologic Oncology, March 2013, Los Angeles, California (oral presentation).

(2) Jackson, A, Zhong, Y, Zhou, C, Kilgore, J, Makowski, L, Gehri,g P, Bae-Jump, V. Metformin had increased efficacy under obese conditions in a novel genetically engineered mouse model of serous ovarian cancer. 45th Annual Meeting of the Society of Gynecologic Oncology, March 2014, Tampa, Florida (poster presentation).

(3) Wysham, WZ, Chen, TH, Makowski, L, Mutch, D, Berchuck, A, Karlan, B, Levine, DA, Bae-Jump, VL, Relationship between Body Mass Index (BMI) and gene expression profiles of high grade serous ovarian cancers in The Cancer Genome Atlas (TCGA) project, 29th Annual Meeting of the Mid-Atlantic Gynecologic Oncology Society, October 2014, Chapel Hill, North Carolina (oral presentation).

(4) Wysham, WZ, Zhang, Y, Dickens, HK, Malloy, KM, Han, XY, Guo, H, Gehrig, PA, Zhou CX, Bae-Jump VL. Differential efficacy of metformin *versus* everolimus in the setting of obesity in a mouse model of serous ovarian cancer. 106th Annual Meeting of the American Association for Cancer Research, April 2015, Philadelphia, Pennsylvania (poster presentation).

(5) Wysham, WZ, Chen, TH, Makowski, L, Levine, D, Mutch, D, Berchuck, A, Karlan, B, Bae-Jump, VL, Relationship between obesity and gene expression profiles of high grade serous ovarian cancers in The Cancer Genome Atlas (TCGA) project, 46th Annual Meeting of the Society of Gynecologic Oncology, March 2015, Chicago, Illinois (poster presentation).

Manuscripts accepted:

(1) Makowski, L, Zhou, C, Zhong, Y, Kuan, PF, Fan, Sampey, BP, Difurio, M and Bae-Jump, VL#. Obesity increases tumor aggressiveness in a genetically engineered mouse model of serous ovarian cancer, Gynecol Oncol, 2014, Apr;133(1):90-7. PMID: 24680597

(2) Stine, JE and Bae-Jump, VL. Metformin and gynecologic cancers. Obstet Gynecol Surv, 2014, Aug; 69(8):477-89. PMID:25144611

Manuscripts submitted:

(1) Guo, H, Zhong, Y, Jackson, A, Clark, L, Kilgore, J, Zhang, L, Han, J, Sheng, X, Gilliam, TP, Gehrig, PA, Zhou, C, Bae-Jump, VL. Everolimus exhibits anti-tumorigenic activity in obesity-induced ovarian cancer, Submitted to *Oncotarget*.

(7) INVENTIONS, PATENTS AND LICENSES: NONE

(8) REPORTABLE OUTCOMES: NONE

(9) OTHER ACHIEVEMENTS

Grants submitted:

NIH/NCI - 1R01CA207838-01
Metabolic and Molecular Biomarkers of Metformin Response in Obesity-Driven Endometrial Cancer
July 2016 – June 2021
$1,250,000 (total direct costs), $250,000 (annual direct cost for year one)
Principal Investigator: Bae-Jump
25% effort (3.0 calendar)
Project Goals: Epidemiological and pre-clinical data suggest that metformin may be efficacious in endometrial cancer. However, two important questions that need to be addressed are: (1) Will metformin be universally

effective in endometrial cancer or be more efficacious in the obese/insulin-resistant patient population? and (2) What role do transporters play in metformin uptake and action in the malignant endometrium? These fundamental questions will be explored in endometrial cancer, a disease driven by obesity and insulin resistance, and key molecular and metabolic biomarkers of metformin responsiveness will be determined, using endometrial cancer mouse models and a phase 2/3 clinical trial in endometrial cancer patients.

Department of Defense/Ovarian Cancer Research Program (DOD/OCRP)
Investigator-initiated Research Award
<u>Circulating Tumor Cells in the Early Detection, Treatment and Management of Ovarian Cancer</u>
January 2016 – December 2018
$448,881 (total direct cost), $147,226 (annual direct cost for year one)
Principal Investigator: Bae-Jump
5% effort (0.6 calendar)
Project Goals: We will evaluate the utility of our novel triple selection circulating tumor cell (CTC) assay through the use of (1) genetically engineered ovarian cancer (OC) mouse models (GEMMs) as a means to provide access to well-defined early and advanced disease blood samples mimicking the human disease and (2) a pilot clinical trial of early and late stage OC patients followed through surgery, chemotherapy and monitoring for disease recurrence. Lastly, we will genetically characterize CTC subpopulations (CTC$^{FAP\alpha}$, CTC^{EpCAM} and CTC^{CSC}) in both mice/women using next generation sequencing as a means to determine the presence of DNA mutations among these CTC subpopulations that are associated with early *versus* late stage disease as well as response and resistance to chemotherapy and disease recurrence.

NIH/NCI - 1R01CA204859-01
<u>Obesity-Induced Metabolic Signature of Ovarian Cancer and Impact on Treatment</u>
April 2016 – March 2021
$1,250,000 (total direct costs), $239,170 (annual direct cost for year one)
Principal Investigator: Bae-Jump
25% effort (3.0 calendar)
Project Aims:Ovarian cancer arising in obese mice is more aggressive and is characterized by a genetic and metabolic profile distinct from tumors developing in lean mice, resulting in increased sensitivity to the anti-tumorigenic effects of metformin. We hypothesize that these obesity-driven alterations are initially established by the nutrient-rich obese host environment and become fixed such that subsequent changes in the metabolic host milieu become irrelevant. This will be explored through (1) delineating a timeline of genetic and metabolomic divergence between ovarian tumors in obese and lean mice using our unique KpB mouse model, (2) via transplantation of ovarian tumors arising in either obese or lean environments to the opposite host phenotype and assessing for metformin responsivity and (3) through an ongoing phase 2 trial of metformin/paclitaxel/carboplatin in ovarian cancer patients.
Note: To be resubmitted in 2016.

Grants awarded:

American Association of Obstetricians and Gynecologists Foundation (AAOGF)
Bridge Funding Award
<u>Obesity, Cation-Selective Transporters and Metformin in Endometrial Cancer</u>
July 2015 – June 2016
$75,000 (total and annual direct costs)
Principal Investigator: Bae-Jump
5% effort (0.6 calendar)
Project Goals: Mounting epidemiological and preclinical data suggest that metformin may be efficacious in endometrial cancer. However, two important questions that need to be addressed are: (1) Will metformin be universally effective in endometrial cancer or be more efficacious in the obese/insulin-resistant patient population? and (2) What role do transporters play in metformin uptake and action in the malignant endometrium? These fundamental questions will be explored in endometrial cancer, a disease driven by

obesity and insulin resistance, using endometrial cancer mouse models and phase 0 and phase 2/3 clinical trials in endometrial cancer patients.

North Carolina Biotechnology Center (NCBC) Collaborative Funding Grant
Discovery of Novel, Efficacious and Safe Biguanides for the Treatment of Ovarian Cancer
July 2015 – June 2017
$180,000 (total direct costs), $90,000 (annual direct costs)
Principal Investigator: Bae-Jump
5% effort (0.6 calendar)
Project Goals: The goal of this project is to assess novel tissue-selective activators of AMP-activated protein kinase (AMPK) (Novatarg) as targeted therapies for the treatment of ovarian cancer using genetically engineered mouse models of serous ovarian cancer.

The North Carolina Translational and Clinical Sciences Institute
NC TraCS $50K Pilot Grant Program
Evaluating the Anti-Proliferative Effects of Atorvastatin on the Endometrium of Endometrial Cancer Patients: A Pre-Operative Window Study
May 2015- April 2016
$50,000 (total and direct costs)
Principal Investigator: Kim
Co-Investigator: Bae-Jump
5% effort (0.6 calendar)
Project Goals: We will conduct a pre-operative window study of atorvastatin in endometrial cancer patients

American Cancer Society
Research Scholar Grant – RSG CCE 128826
Obesity, Cation-Selective Transporters and Metformin in Endometrial Cancer
January 2016 – December 2019
$659,626 (total direct costs), $164,963 (annual direct cost for year one)
Principal Investigator: Bae-Jump
20% effort (2.4 calendar)
Project Goals: We will evaluate the contribution of a representative metformin transporter to the anti-tumor efficacy of metformin in obese and non-obese orthotopic mouse models, in which tumors are derived from an endometrial cancer cell line with normal or overexpression of a representative transporter. In addition, we will correlate treatment response to metformin in EC patients with (i) expression and genetic variants of the metformin transporters, (ii) modulation of the AMPK-mTOR pathway, and (iii) metabolic factors associated with obesity.

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PRECLINICAL AND CLINICAL INVESTIGATION OF THE IMPACT OF OBESITY ON OVARIAN CANCER PATHOGENESIS

PARTICIPANTS

This DOD/OCRP Translation Pilot Award application is synergistic, requiring and capitalizing on the skills of multidisciplinary participants. Dr. Bae-Jump is a gynecologic oncologist and translational cancer researcher with extensive experience in the investigation of targeted therapies in ovarian and endometrial cancer. Dr. Bae-Jump will serve as Principal Investigator for this project. She has assembled an experienced team of outstanding researchers and collaborators, creating a unique environment to study the impact of obesity on ovarian cancer pathogenesis and the potential use of mTOR inhibitors and metformin as a chemotherapeutic strategy in this disease. Dr. Makowski has expertise and training in obesity research, metabolism, lipid biology, and inflammation. She will serve as a Co-Investigator for this project. By bringing together experienced researchers and collaborators with diverse and complementary skills, we will increase our efficiency to ensure our collective success. The work proposed would be a new project for our laboratories and cannot be done without the support of the DOD/OCRP funds.

Victoria Bae-Jump, M.D., Ph.D., Principal Investigator, (1.2 cal months, 10% effort, no salary requested). Dr. Bae-Jump is an Assistant Professor in the Division of Gynecologic Oncology. She is a gynecologic oncologist and a translational cancer researcher at the Lineberger Comprehensive Cancer Center (LCCC) in the Clinical Research Program. Dr. Bae-Jump received her PhD and MD and did a postdoctoral fellowship at Virginia Commonwealth University. She performed her residency and fellowship at UNC Chapel Hill in Obstetrics & Gynecology and Gynecologic Oncology. Dr. Bae-Jump joined the faculty at UNC in July 2007 and was awarded an institutional Multidisciplinary Clinical Research Career Development Roadmap K12 grant. In September of 2010, she subsequently received a 5 year NIH/NCI K23 Mentored Patient-Oriented Research Career Development Award. Her research focuses on understanding the interactions between cell signaling pathways implicated in endometrial and ovarian cancer pathogenesis as a means to target therapy for this disease. In this pursuit, she has investigated many novel targeted therapies for the treatment of endometrial and ovarian cancer, including mTOR inhibitors, arsenic, a human monoclonal antibody to the insulin growth factor-1 receptor (IGF-1R), soy, genistein and most recently, metformin. In addition, she has a clinical trial underway that is a preoperative window study of metformin in obese endometrial cancer patients. She has also expanded her interests to include the development of an ovarian cancer mouse model that will be utilized in this project. Dr. Bae-Jump has expertise in molecular and cellular biology, animal and cell culture studies as well as microarray and immunohistochemical analysis for the exploration of alterations in cell signaling pathways in gynecologic malignancies. Dr. Bae-Jump will oversee the experimental design, implementation and analysis of this project and will directly interact with her research associate, Dr. Chunxiao Zhou. **Dr. Bae-Jump is funded by a K23 grant which covers the scope of the work; therefore, no additional salary support is requested.**

Liza Makowski Hayes (a.k.a. Liza Makowski), M.M., Ph.D., Co-Investigator (0.60 cal months, 5% effort, no salary requested). Dr. Makowski is an Assistant Professor of Nutrition, a member of the Nutrition Obesity Research Center, Center for Gastrointestinal Biology and Disease, McCallister Heart Center, and Lineberger Comprehensive Cancer Center at UNC Chapel Hill. Dr. Makowski's Ph.D. in the Department of Nutrition at the Harvard School of Public Health with Dr. Gökhan Hotamisligil focused on how fatty acid transporters modulate the macrophage inflammatory response and cholesterol metabolism in atherosclerosis, for which she received NRSA and NIH-LRP fellowships. She also obtained a Masters in Medicine as a Lucille Markey Fellow from Harvard Medical School during her Ph.D. studies. Her postdoctoral studies under Drs. Deborah Muoio and Chris Newgard in the Departments of Medicine and Pharmacology & Cancer Biology utilized animal and cell culture models in combination with comprehensive metabolomic profiling at the Duke Stedman Center for Nutrition and Metabolism to address the role of mitochondrial fuel metabolism in times of overnutrition. She currently holds a K99/R00 to investigate the role of macrophage lipid metabolism and inflammation in obesity, and a U01 to examine the effect of partum obesity on breast cancer focusing on the inflammatory

microenvironment. She has expertise in mitochondrial metabolism, inflammation, microarray and metabolomic analysis, and histological analysis in animal and cell culture-based obesity, cancer, and diabetes/insulin resistance research . Dr. Makowski will supervise Dr. Freemerman in the design of all experiments, review and guide the interpretation of all data, assist as needed in experimental procedures, and aid in preparation of the manuscript. **Dr. Hayes' effort will be covered by institutional funds.**

Chunxiao Zhou, M.D., Ph.D., Research Associate (6 cal months, 50% effort, only request 35% effort). Dr. Zhou is the laboratory manager and lead research associate for Dr. Bae-Jump's lab. Dr. Zhou has a M.D., Ph.D. awarded in China. He joined the Division of Gynecologic Oncology at UNC-CH in 2000 as a postdoctoral research associate and has worked in the laboratory of Dr. Bae-Jump since July 2007. He has a vast range of experience with molecular and cellular techniques, including *in vitro* gene expression studies, PCR, electrophoresis, cell-based assays, SDS-PAGE, Western immunoblotting, immunhistochemical techniques as well as animal/mouse work. **He will contribute 50% of his time to this project. 35% salary support is requested and the remaining 15% will be covered by institutional funds.**

Alex J. Freemerman, Ph.D., Research Associate/Lab Manager (2.4 cal months, 20% effort). Dr. Freemerman is the laboratory manager and lead research associate for Dr. Makowski's lab. He holds a Ph.D. in Cell Biology and has more than 15 years of experience in bench research focused on pre-clinical cancer drug discovery, including 10 years as a Research Assistant Professor in the Department of Surgery at Duke University Medical Center studying xenografts in mouse models. Dr. Freemerman has expertise in examining macrophage and adipocyte-derived stem cell signaling cascades, metabolic biochemical assays, as well as considerable experience in managing rodent colonies, culturing primary cells, minor rodent surgery, and immunohistochemistry. **He will commit 20% of his time to this project.**

Wei Jia, Ph.D., Co-Director, NORC Metabolomics Core (0 cal months, 0% effort). Dr. Wei Jia is a Professor in the Department of Nutrition and the Director of the UNCG Center for Research Excellence in Bioactive Food Components at our Kannapolis Campus. He also serves as the Co-Core Director for the NORC Metabolomics Core. Dr. Jia is an expert in metabolomic studies and profiling and his research interests lie in the area of identifying metabolite markers for disease, studying baseline metabolic signatures to predict how individuals will respond to a specific nutritional intervention and, in addition, understanding how bioactive phytochemical compounds interact with metabolic pathways. As Director of the UNCG Center for Research Excellence in Bioactive Food Components, Dr. Jia is responsible for assisting researchers with human studies requiring metabolic profiling. He directs a state-of-the-art facility that includes a metabolomic profiling platform featuring an Agilent HPLC TOF-MS system and Leco GC-TOF-MS system. Dr. Jia provides metabolite expression patterns in both human and animal samples for NORC users. Dr. Jia will supervise the metabolomics data collection and analysis and be actively involved in the elucidation of biochemical pathway perturbations involved in obesity and ovarian cancer pathogenesis as well as biomarkers of mTOR inhibitor and metformin treatment both in human specimens and the KpB mouse model. **Dr. Jia is already supported for this Core Resource activity.**

Xiuxia Du, PhD, Bioinformatics Concierge, NORC Metabolomics Core (0 cal months, 0% effort). Dr. Du is an Assistant Professor of Bioinformatics at UNC-Charlotte, and the Bioinformatics Concierge for the NORC Metabolomics Core. She received her Ph.D. in Systems Science and Mathematics from Washington University in St. Louis in 2005. She subsequently did her post doctorate research in Dr. Richard D. Smith's lab at the Pacific Northwest National Laboratory. Her research is focused on developing bioinformatic approaches for integrating metabolomic and nutrigenomic data analyses. Dr. Du works with NORC members who are conducting systems biology nutrition research helping them to use and develop bioinformatic tools in analyzing this complex data. Her office is located at our Nutrition Research Institute (NRI) Kannapolis campus. She will assist with the metabolomics data analysis and be actively involved in the elucidation of biochemical pathway perturbations involved in obesity and ovarian cancer pathogenesis as well as biomarkers of mTOR inhibitor and metformin treatment both in human specimens and the KpB mouse model. **Dr. Du is already supported for this Core Resource activity.**

Cheng Fan, MS, Bioinformatics/Statistician (0.24 cal months, 2% effort, no salary requested). Cheng Fan, MS (UNC-CH) from the Genomics/Bioinformatics Core Facility will serve as the Bioinformatics/Statistician for this work. He has a Masters Degree in both Computer Information Science and Computational Biology, and joined the Lineberger Comprehensive Cancer Center in 2004 as a Bioinformatics Research Associate. Cheng Fan has extensive experience in analyzing DNA microarray expression data for a variety of cancers, most notably breast cancer. He has developed statistical strategies for analyzing microarray datasets associated with a multitude of cancers and has provided consultations for many researchers in experimental design and data analysis in regards to gene expression profiling and data mining. **Cheng Fan's salary is provided by Lineberger Comprehensive Cancer Center, and thus, salary support is not requested for his contribution to this project.**

Pei-Fen Kuan, Ph.D. Metabolomics Biostatistical support (0.24 cal months, 2% effort, no salary requested). Dr. Kuan is a Research Assistant Professor, in the Department of Biostatistics and is a member of the Lineberger Comprehensive Cancer Center Biostatistics Core. Her research focuses on computational biology to examine Chip on CHIP data statistical genomics as well as most other genomic data using primarily clinically-derived samples. In collaboration with Dr. Makowski, Dr. Kuan has analyzed a large data set of tissue and plasma metabolites for a diet-induced obesity study. Through multiple Spearman pairwise correlations, we were able to identify significant metabolite and gene expression correlations with relevant physiologic findings using a Benjamini-Hochberg false discovery rate in the software package R. This allowed Dr. Makowski to focus on the strongest and putatively most biologically relevant metabolomic and genomic findings (manuscript in preparation). For this project, she will provide biostatistical support in order to define metabolic pathways that are involved in response to mTOR inhibitor and metfromin treatment in ovarian cancer as well as the impact of the obese environment on sensitivity to these agents. **Dr. Kuan's salary is provided by Lineberger Comprehensive Cancer Center, and thus, salary support is not requested for h contribution to this project.**

OTHER SIGNIFICANT CONTRIBUTORS
COLLABORATORS

Charles Perou, Ph.D., Collaborator (0 cal months, 0% effort). Dr. Perou is an Associate Professor of Genetics and Pathology and Laboratory Medicine and is Scientific Director of the UNC-CH Genomics and Bioinformatics Core Facility and Co-Director of the Mouse Phase I Unit (MPIU). He is a leading figure in cancer gene expression profiling, and is Co-Director of the The Cancer Genome Atlas (TCGA) project at UNC-CH. UNC-CH was one of twelve centers chosen for this large-scale, collaborative effort by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI). Their goal is to systematically characterize the genomic changes that occur in various cancers. The focus of Dr. Perou's laboratory is the characterization of the biological diversity of human tumors using genomics, molecular genetics, and cell biology. He is directly involved in the translation of these technologies to develop improved diagnostics and therapies that are specific for each cancer subtype. As further testament to his expertise, Dr. Perou won the 2009 American Association for Cancer Research (AACR) Outstanding Investigator Award in Breast Cancer for his work on deciphering the underlying biology of distinct molecular subtypes of breast cancer. Dr. Perou and Dr. Bae-Jump have ongoing collaborations in the microarray analysis of both ovarian and endometrial cancer tumors. Dr. Perou also collaborates with Dr. Makowski on her U01 breast cancer grant. Dr. Perou's role will be to assist Dr. Bae-Jump in the analysis of the microarray gene expression data derived from ovarian cancer specimens from obese and non-obese women and KpB mice, especially in regards to the cross-species comparisons. In addition, Dr. Perou will provide the resources and expertise of the Mouse Phase I Unit for the implementation of the studies on metformin and everolimus stratified for obesity in the KpB ovarian cancer mouse model.

D. Neil Hayes, M.D., Ph.D., Collaborator (0 cal months, 0% effort). Dr. Hayes is an Associate Professor of Hematology Oncology and is an expert on clinical and translational genomics of aerodigestive tumors. At UNC,

he has generated genomic and array data on over 1000 patients, including gene expression arrays, SNP chips, methylation profiles, tissue microarrays, and DNA sequencing. Techniques employed include genomic profiling, targeted sequencing, and high-resolution shotgun sequencing. He has participated in method development for genomic analysis including hypothesis testing, high-throughput sequence analysis, and quality assessment and has designed and executed numerous clinical cancer therapy trials. Dr. Hayes is the Clinical Director of Bioinformatics for the Lineberger Comprehensive Cancer Center, with primary oversight for their clinical and genomic data systems and is the Medical Director of the UNC Hospitals Tumor Registry. Dr. Hayes also serves as Co-PI for UNC's participation in the Cancer Genome Atlas (TCGA), a pivotal project of the National Institutes of Health. Dr. Hayes will work with Dr. Bae-Jump and her lab for the optimization of the tissue microarrays and immunohistochemical protocols that are to correlate expression of key components of the glucose metabolism pathway and the closely related IGF-1R and PI3K/Akt/mTOR pathways with ovarian tumors derived for obese versus lean women and mice.

(11) APPENDICES: None